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Functional significance of the copper transporter ATP7 in peptidergic neurons and endocrine cells in *Drosophila melanogaster*

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ABSTRACT

The *Drosophila* ATP7 copper transporter has sequence homology to the human copper transporters ATP7A and ATP7B, which are defective in Menkes and Wilson disease, respectively. We show here that in *Drosophila* ATP7 is expressed by many peptidergic neurons. As C-terminal amidation of neuropeptides depends on the copper-containing enzyme PHM, it seemed likely that in the absence of ATP7 the activity of PHM might be compromised. Indeed, inhibition of ATP7 expression by RNAi led to a decrease in mature amidated neuropeptides and the appearance of C-terminally Gly-extended neuropeptides. The strength of this effect differed from one cell type to another; it was very pronounced for AKH and corazonin, but much less so for SIFamide and myosuppressin. Nevertheless, down-regulation of ATP7 specifically in the SIFamide-expressing neurons resulted in male–male courtship behavior.

1. Introduction

Copper is an essential cofactor for a variety of enzymes while at the same time it is toxic due to its generation of free radicals [1]. Consequently, copper homeostasis is tightly regulated by a number of specific membrane transporters, thus allowing uptake of needed copper and elimination of excess. The P-type ATPase copper transporters ATP7A and ATP7B are regulators of intracellular copper homeostasis, and are normally located in the trans-Golgi network where copper is imported and becomes incorporated into copper-dependent enzymes. When intracellular copper concentrations increase too much, ATP7-A and -B relocate and assure the elimination of copper from the cell [2]. When one of the copper homeostatic proteins fails, problems arise. Menkes disease is an often fatal human disease characterized by cerebral and cerebellar neurodegeneration in which the ATP7A transporter is defective [3], while patients suffering from Wilson disease have a defective ATP7B transporter, leading to an accumulation of intracellular copper, particularly in the liver and in the brain [4].

It has been noted that many of the proteins involved in copper homeostasis are well conserved between mammals and insects [5–9]. The ATP7A and ATP7B copper transporters are represented by a single homolog in *Drosophila melanogaster*, ATP7 [9]. ATP7 is abundantly expressed in the copper cells in the midgut, acid secreting cells known to produce metallothionein when copper is abundant in the food [10], but also in neurons in the central nervous system [11]. The neuronal distribution suggests that at least some of the ATP7 expressing cells might produce C-terminally amidated neuropeptides. C-terminal amides in neuropeptides are produced by the sequential action of two enzymes, a peptidylglycine-α-hydroxylating mono-oxygenase (PHM) and a peptidyl-α-hydroxyglycine lyase (PAL), which in vertebrates are combined into one bifunctional enzyme called PAM (peptidylglycine α-amidating monooxygenase), but which in *Drosophila* are encoded by different genes [12]. Since PHM is dependent on copper [13] the putative expression of ATP7 in cells producing amidated peptides suggests that its presence in these cells is necessary to make copper available for PHM. ATP7 is not the only protein important for copper metabolism. Thus copper is imported into the cell by Ctr1 proteins, encoded in *Drosophila* by three genes, *ctr1A, ctr1B* and *ctr1C* [5–9], and it has previously been suggested that *ctr1A* is essential for C-terminal amidation of FMRFamides in the fruit fly [14].

Whereas in mouse models for Menkes disease the ATP7A transporter is non-functional in the entire animal, in *Drosophila* it is relatively easy to specifically diminish ATP7 expression in restricted subsets of neurons using RNAi and the binary UAS-gal4 expression system [15]. We here describe that RNAi-mediated down regulation of ATP7 in specific peptidergic cells leads to a decrease

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in C-terminal amidation of neuropeptides, but that the effect is strikingly different among different neuropeptidergic cell types.

2. Materials and methods

2.1. Flies

Flies were raised at 25 °C on standard corn meal food in a 12 h dark/light cycle. Low copper food was prepared by adding 10 μM of the copper chelator tetrathiomolybdate (Sigma–Aldrich, Saint-Quentin Fallavier, France) to the food. The AKH-gal4, SIFa-gal4, Crz-gal4 and MS-gal4 transgenic flies, which express gal4 specifically in the adipokinetic hormone, SIFamide, corazonin and myosuppressin-expressing cells, respectively, have been previously described [16–19]. The UAS-ATP7-RNAi stock w^{1118}; P{GD3322}v8315 [20] was from the Vienna Drosophila RNAi Center and the UAS-ATP7-RNAi stock y^1 v^1; P{TRiP.JF01534}attP2, UAS-LacZ and UAS-Dcr2 were from the Bloomington stock center. The two ATP7-RNAi lines target different parts of the coding sequence of the ATP7 gene (Fig. S1). The ATP7-gal4 line [11] was a kind gift from Dr. Richard Burke (Monash University, Victoria, Australia).

2.2. Immunohistology and antisera

Rabbit PHM and DIMM antisera were generously provided by Prof. Paul Taghert (Washington University, St. Louis, USA). Immunohistological procedures and other antisera used are those described elsewhere [21].

2.3. Male–male courtship behavior

Flies used in male–male courtship behavior essays were isolated within 6 h of emergence and kept isolated until use. Three-to-five day old males to be tested were aspirated into a courtship chamber of 4 mm by 22 mm diameter with a wild type male. The percentage of time during a 10 min observation period spent in courtship behavior (i.e. orientation, following, tapping, wing extension, licking and (attempted) copulation) was defined as the courtship-index [22]. Wing extension was measured separately to confirm the sexual intensity of the observed behavior.

2.4. Direct MALDI-TOF mass spectrometric peptide profiling

The availability of heavy-isotope labeled *Drosophila* AKH* (pGlu-Leu[$^{13}C_6$,^{15}N]-Thr-Phe-Ser-Pro-Asp-Trp-amide, custom-made by Iris Biotech, Marktredwitz, Germany, Mw = 982.5 Da, i.e. 7 Da higher than than that of the naturally occuring AKH) and myosuppressin* (Thr–Asp–Val[d8]–Asp–His–Val–Phe–Leu–Arg–Phe–amide, custom-made by Biosyntan, Berlin, Germany, Mw = 1255.4 Da, i.e. 8 Da higher than the naturally occuring myosuppressin) made it possible to directly semi-quantify the amounts of AKH and myosuppressin in the the adult CC/hypocerebral ganglion complex using direct mass-spectrometric tissue profiling as described previously [23,24]. Such mass spectrometry standards are not available for SIFamide or corazonin, and for these peptides we employed the isotopically labeled myosuppressin* as a standard. We quantified both the mature amidated peptide and the substrate for PHM, i.e., the neuropeptide extended with a C-terminal Gly residue, relative to the isotopically labeled standards. Retrocerebral complexes of adult flies were punched out with pulled glass capillaries and spotted onto the MALDI target for analysis of myosuppressin, corazonin and AKH. For the analysis of SIFamide brain regions were dissected and analyzed. Matrix consisted of a saturated solution of recrystallized α-cyano-4-hydroxycinnamic acid in MeOH/EtOH/H_2O 30/30/40%, and 200 nl was added to each sample with a micropipette. MALDI-TOF mass spectra were acquired in positive ion reflectron mode on an Applied Biosystems Voyager 4800 + MALDI TOF/TOF mass spectrometer. Matrix ions were suppressed by setting the low mass gate at 850 Da. To obtain optimal signal-to-noise ratios laser power was adjusted on one sample and then maintained constant for the others on the same sample plate. Each spectrum consisted of ten to twenty subspectra with 50 shots each. Data were analyzed with Data Explorer 4.3 software (ABSciex). For quantification, the relative peak intensities for the different adducts ($[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$) of each peptide were summed after the mass spectra had been base-line corrected and de-isotoped; subsequently the ratios of the resulting relative peak intensities of the ions of interest (AKH/AKH*, AKH-Gly/AKH*, MS/MS*, MS-Gly/MS*, etc.) were calculated.

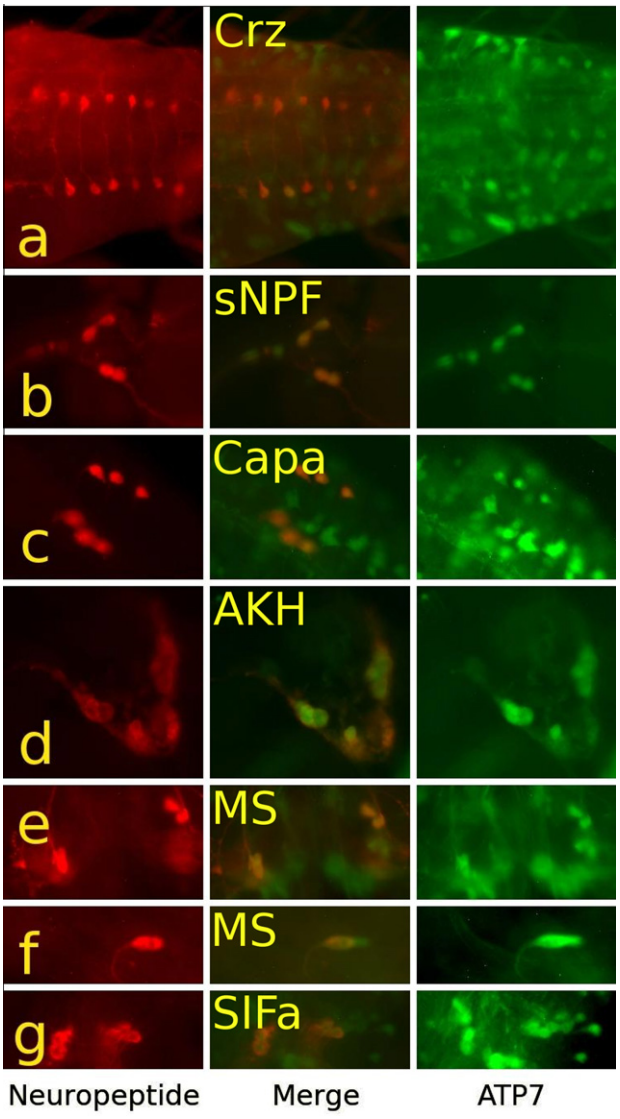

Fig. 1. Colocalization of various neuropeptides in third instar *Drosophila melanogaster* expressing ATP7-gal4-driven LacZ. In each row the picture to the left shows peptide immunoreactivity in red, the one to the right LacZ immunoreactivity in green, and a merged image is shown in the middle. (a) corazonin interneurons in the abdominal neuromeres; (b) sNPF neurons of the hypocerebral ganglion; (c) capa neuroendocrine cells (Va neurons) in the abdominal neuromeres; (d) AKH endocrine cells in the retrocerebral complex; (e) MS neuroendocrine cells in the pars intercerebralis; (f) MS peripheral neuroendocrine cells in the abdomen; (g) SIFamide neurons in the pars intercerebralis.

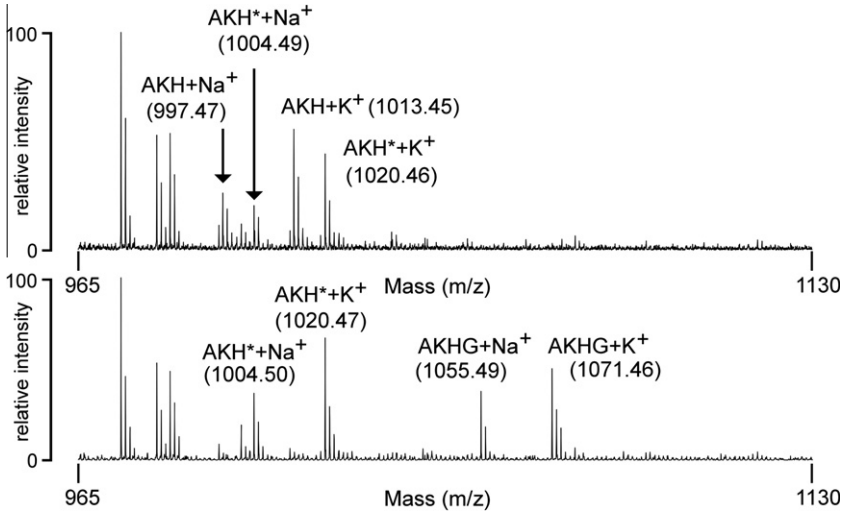

Fig. 2. The top panel shows the mass profile of a retrocerebral complex from a control fly (;;UAS-ATP7-RNAi/+). The characteristic AKH adducts $[M+Na]^+$ and $[M+K]^+$ are as prominently present as are the same adducts of the stable isotope-labeled AKH standard (AKH^*). The bottom panel shows the mass profile of a retrocerebral complex from a fly in which ATP7 was inhibited (UAS-Dcr2/y;AKH-gal4/+;UAS-ATP7-RNAi/+). Here there are no AKH adduct ions, even though the AKH^* standard is easily detected; instead there are Na^+ and K^+ adduct ions for the Gly-extended AKH.

2.5. Statistics

The hypotheses tested were that the combination of the three transgenes – neuropeptide-gal4, UAS-ATP7-RNAi and UAS-Dicer2 – would lead to larger courtship and wing extension indexes, smaller quantities of amidated neuropeptides and increased quantities of Gly-extended neuropeptides. As the data were not normally distributed, we used the Wilcoxon rank-sum test to establish statistical significance. Flies having a copy each of the neuropeptide-gal4 and UAS-Dicer2 transgenes were pooled with the data from flies having a single copy of the UAS-ATP7-RNAi transgene and compared with flies carrying all three transgenes.

3. Results and discussion

To test whether ATP7 is expressed in peptidergic cells, we performed double immuno-stainings for several types of peptidergic cells. In ATP7-gal4/UAS-LacZ flies, LacZ immunoreactivity co-localized with immunoreactivity to myosuppressin, corazonin, SIFamide, capa, leucokinin, AKH and sNPF (Fig. 1). Although we have not systematically tested every single C-terminally amidated *Drosophila* neuropeptide for neuronal colocalisation of ATP7, those that we did test were found to co-localize. However, not all investigated peptidergic cells were equally clearly expressing gal4 under the control of the ATP7 promotor. For example, in some flies not all of the relatively small corazonin interneurons in the abdominal neuromeres were labeled; such individual variability was common for smaller neurons, but occasionally also observed for larger cells. Such differences might be due to temporal patterns of expression, problems in detecting very low levels of expression in small neurons, or because the expression of the transgene does not entirely reflect expression of ATP7 itself. Nevertheless, not all ATP7 expressing neurons are peptidergic, as copper is also an essential cofactor for dopamine β-monooxygenase [25] and ATP7 is thus likely also expressed in aminergic neurons such as those producing octopamine.

Our results suggest a frequent colocalisation of ATP7 and neuropeptides. Since PHM depends on copper, we hypothesised that the expression of ATP7 is needed for biological activity of PHM and that in its absence, neuropeptides might be no longer become C-terminally amidated. We observed that a large number of the ATP7 expressing neurons were also immunoreactive with antiserum to *Drosophila* PHM [12], but we were unable to demonstrate that all PHM neurons express ATP7 (data not shown). We do not know whether this is caused by weak ATP7-gal4-driven reporter expression, or whether some PHM neurons do not express ATP7. There are also several neurons that express ATP7 but are not recognized by the PHM antiserum. Some of these may well be octopaminergic based on their location. Typically, antisera to C-terminally amidated neuropeptides recognize the C-terminal amide, and do not or only weakly recognize the intermediate neuropeptide products, such as those which have C-terminal Gly or Gly-Lys-Arg extensions. Our first attempts were thus directed at differences in immunoreactivity detectable with our various antisera. We compared flies with wild-type levels of ATP7 expression with those in which ATP7 expression had been suppressed via RNAi (see below). In a few preparations the staining intensity appeared to be reduced in flies expressing RNAi in myosuppressin neurons, but not in all specimens and only when larvae were grown on low copper food.

To confirm that the expression of ATP7 RNAi inhibits PHM activity, we used mass spectrometry to analyze whether downregulation of ATP7 by RNAi inhibited the C-terminal amidation of neuropeptides. We thus expressed ATP7 RNAi in neuroendocrine cells and/or neurons that express either adipokinetic hormone (AKH), corazonin (Crz), myosupressin (MS) or SIFamide. In order to maximize the effects of the ATP7 RNAi, a UAS-Dicer 2 transgene was also added and flies were raised on a low copper diet.

In UAS-Dcr2/+;AKH-gal4/+;UAS-ATP-RNAi/+ flies, amidated AKH was virtually absent and instead large quantities of AKH containing a C-terminal Gly residue were observed. Gly-extended AKH was found neither in UAS-Dcr2/+;AKH-gal4/+ nor in;;UAS-ATP-RNAi/+ flies, which contained normal quantities of AKH (Figs. 2 and 3). Similar results were obtained in the case of corazonin. In UAS-Dcr2/+;Crzn-gal4/+;UAS-ATP-RNAi/+ flies amidated corazonin was only found in 2 out of 28 flies and large quantities of Gly-extended corazonin were found in all but one of them. In contrast, amidated corazonin was found in all UAS-Dcr2/+;corazonin-gal4/+ and;;UAS-ATP-RNAi/+ flies, and Gly-extended corazonins was rarely present in these flies (Fig. 3). The decrease in mature peptide and the increase in Gly-extended neuropeptides are statistically highly significant (all P's < 0.0001) for both AKH and corazonin. To exclude the possibility that the observed effects are due to off-target gene regulation by RNAi, we repeated these experiments

with a second UAS-RNAi gene which is directed toward a different part of the ATP7 gene, and found the same results (Fig. S2).

For both myosuppressin and SIFamide, the decrease in the quantities of mature peptides was much less dramatic, and Gly-extended neuropeptides were only found in a fraction of the tested flies (Fig. 3). The difference in Gly-extended neuropeptides between control and experimental flies barely reached significance for SIFamide ($P < 0.05$), and was not statistically different from controls for myosuppressin. Still, in some experimental flies, but not in controls, large amounts of SIF-Gly were found, showing that the

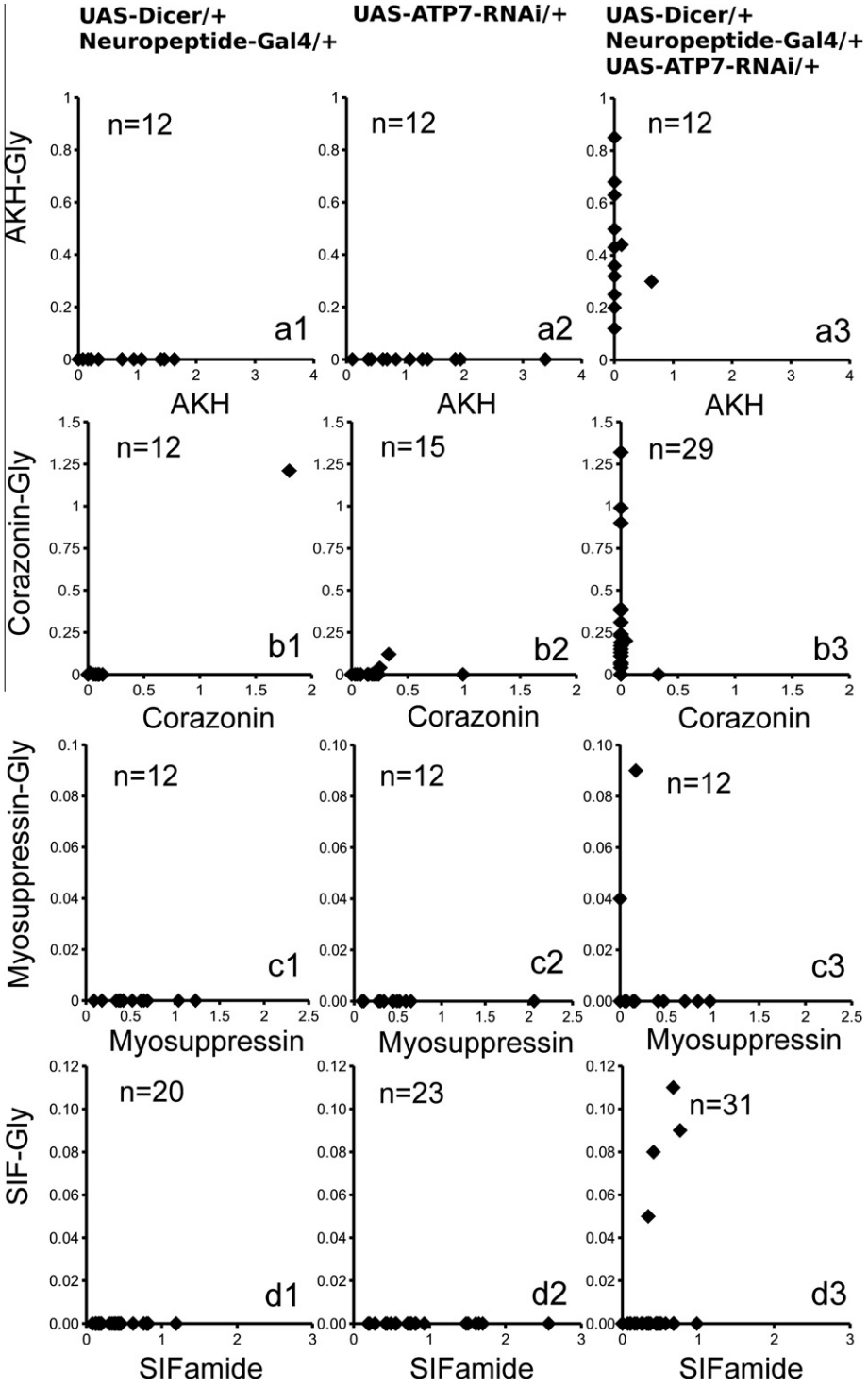

Fig. 3. Plots showing the relative concentration (measured as ratio relative to the stable isotopically labeled AKH* or myosuppressin* standards) of mature amidated peptide on the X-axis and its C-terminally extended Gly analog, the PHM substrate, on the Y-axis. a1–a3, AKH, b1–b3, corazonin, c1–c3, myosuppressin and d1–d3 SIFamide. Note that with a single outlier in b1 significant quantities of Gly-extended neuropeptides are only found in a3, b3, c3 and d3, i.e., in those flies in which ATP7 was inhibited by RNAi in the specific cell type. The effects are very pronounced in the AKH and corazonin cells, but much less so in the myosuppressin and SIFamide neurons. Genotypes: **a1:** UAS-Dcr2/+;AKH-gal4/+, **a2:**;;UAS-ATP7-RNAi/+, **a3**: UAS-Dcr2/+;AKH-gal4/+;UAS-ATP7-RNAi/+, **b1:** UAS-Dcr2/+;Crzn-gal4/+, **b2:**;;UAS-ATP7-RNAi/+, **b3:** UAS-Dcr2/+;Crzn-gal4/+;UAS-ATP7-RNAi/+, **c1:** UAS-Dcr2/+;MS-gal4/+, **c2:**;;UAS-ATP7-RNAi/+, **c3:** UAS-Dcr2/+;MS-gal4/+;UAS-ATP7-RNAi/+, **d1:** UAS-Dcr2/+;UAS-SIFa-gal4/+, **d2:**;;UAS-ATP7-RNAi/+, **d3:** UAS-Dcr2/+;UAS-SIFa-gal4/+;UAS-ATP7-RNAi/+.

suppression of ATP7 did effect PHM activity. Nevertheless, the flies which had significant quantities of SIF-Gly still produced some SIFamide, indicating that the effects of ATP7 RNAi in the SIFamide neurons were not as pronounced as in AKH- or corazonin-expressing cells.

Elimination of the SIFamide neurons by genetic ablation or suppression of SIFamide synthesis by RNAi induces male-male courtship behavior in *Drosophila* [17]. We thus anticipated that if the expression of ATP7 RNAi by the SIFamide neurons were able to significantly reduce the amounts of SIFamide produced by these neurons, it might induce male–male courtship behavior. UAS-Dcr2/y;SIFamide-gal4/+;UAS-ARNi-ATP7/+ flies propagated on normal fly food showed no male-male courtship behavior, but when the same experiment was repeated with flies raised on food with a copper chelator male–male courtship behavior was observed (Fig. 4). However, although both the courtship and the wing extension indices were statistically significantly higher in flies in which ATP7 expression was suppressed by RNAi ($P < 0.001$ and $P < 0.02$, respectively), male–male courtship behavior only occurred in a subset of males, and with a courtship intensity clearly less pronounced than in males either lacking SIFamide neurons or in which expression of the SIFamide gene was silenced by RNAi [17]. The fractions of male flies showing male–male sexual behavior was similar to the fraction of flies showing a perturbed processing of SIFamide, and these data are thus concordant.

ATP7 RNAi is unlikely to be 100% efficient in suppressing the expression of ATP7 and in the presence of high copper concentrations small amounts of ATP7 are likely sufficient to insure functional PHM in peptidergic neurons. This is similar to Menkes patients, who have mutant ATP7A transporters retaining residual copper transport activity. In such patients disease can be avoided by subcutaneous injection of copper complexes, showing that increased copper concentration can compensate a faulty ATP7A transporter [3]. It is therefore not surprising that the use of a copper chelator to decrease copper concentration makes it easier to demonstrate the effects of a diminished ATP7 expression in *Drosophila* peptidergic neurons.

Peptidergic neurons release peptides by exocytosis, and when this occurs PHM, which is bound to the membrane of the secretory granule, is exposed to the extracellular medium until it is reinternalized by endocytosis. As the copper ion is not tightly bound [26], this is an occasion where it may be readily lost from the enzyme. Rapid turnover rates of peptides might thus be associated with concomitantly high turnover rates of copper, but whereas the cellular machinery can handle high peptide turnover rates, in the absence of normal quantities of ATP7 it might not be able to replenish the copper sufficiently fast. Thus diminished ATP7 expression might have stronger effects in actively secreting cells and this might explain why the effects of ATP7 RNAi were more robust on the AKH and corazonin neuroendocrine cells as compared to the myosuppressin and SIFamide cells. Furthermore, in actively secreting cells with a high rate of peptide synthesis, the production of gal4 is likely to be higher than in quiescent cells, since peptide synthesis is activated by the same promotor that drives gal4-expression. Thus in actively secreting cells the expression of ATP7 RNAi is expected to be increased and this may provide an additional explanation as to why the effects of ATP7 RNAi may be more pronounced in actively secreting cells. Although it could also be argued that the gal4 drivers used for the AKH and corazonin cells are stronger than those used in the SIFamide and myosuppressin neurons, we feel this is unlikely, as apoptosis induced by UAS-reaper in the various neurons follows similar time scales (unpubl. data).

In the case of a genetic defect in a widely expressed protein like ATP7A it is not easy to pinpoint where exactly diminished enzyme activity causes pathological problems. Indeed, the same genetic defects can lead to different symptoms. It has previously been shown that in mottled brindled mice, a murine model for Menkes disease, neuropeptide amidation is reduced, implying a reduction in PHM activity [26,27]. However, technical difficulties made it impossible to demonstrate differences in PHM activity [27]. By diminishing expression of ATP7 specifically in four different peptidergic cell types using *Drosophila* genetics, we were now able to show that in all four cell types neuropeptide amidation is perturbed, but that the degree is different from one cell type to another, even though the direct effect, i.e., lack of sufficient copper for PHM, is likely the same. Even in genetically identical individuals we did not always see the same phenotypes, as in the case of SIFamide and myosuppressin. Similar differences in Menkes patients could explain why symptoms vary from patient to patient.

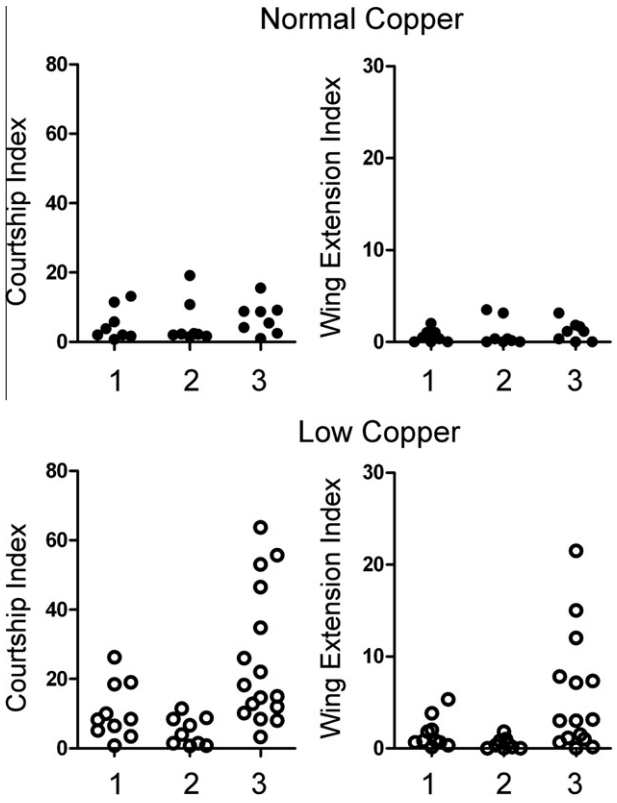

Fig. 4. Courtship and wing extension indices of males toward males. The courting males have the following genotypes: **1:** UAS-Dcr2/y;SIFa-gal4/+, **2:**;;UAS-ATP7-RNAi/+ and **3:** UAS-Dcr2/y;SIFa-gal4/+;UAS-ATP7-RNAi/+; flies were raised on normal fly food (top panels) or on fly food containing a copper chelator (bottom pannels). Note that some, but not all, males of the UAS-Dcr2/y;SIFa-gal4/+;UAS-ATP7-RNAi/+ genotype show male–male courtship behavior, but only when raised on low copper food. Courtship and wing extension indices of the UAS-Dcr2/y;SIFa-gal4/+;UAS-ATP7-RNAi/+ under low copper conditions are significantly different from those of the other flies ($P < 0.001$; $P < 0.02$).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.08.009.

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